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Variability of genes encoding nonstructural proteins of rotavirus A (Reoviridae: *Rotavirus: Rotavirus A*) genotype G9P[8] during the period of dominance in the territory of Nizhny Novgorod (central part of Russia) (2011–2020)

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Introduction. In Russia, rotavirus A is the main cause of severe viral gastroenteritis in young children. The molecular features that allow a rotavirus of a particular genotype to gain an evolutionary advantage remain unclear, therefore, the study of the genetic diversity of rotaviruses based on genes encoding nonstructural proteins (NSPs) responsible for the reproduction of the virus in the cell is an urgent task.
Objective. To study the genetic diversity of rotaviruses of genotype G9P[8], which dominated Nizhny Novgorod in 2011–2020, based on genes encoding nonstructural proteins.
Materials and methods. Rotavirus-positive samples were subjected to PCR-genotyping and sequencing of *NSP1–NSP5* genes. Phylogenetic analysis was carried out in the MEGA X program.
Results. In the period 2011–2020, G9P[8] rotaviruses with four variants of the *NSP2* gene were co-circulating in Nizhny Novgorod. New alleles were noted in 2012 (N1-a-III), 2016 (N1-a-IV) and in 2019 (N1-a-II). The appearance of new variants of other genes occurred in 2014 (E1-3, *NSP4*), 2018 (T1-a3-III, *NSP3*) and in 2019 (A1-b-II, *NSP1*). *NSP2* gene had the most variable amino acid sequence (16 substitutions), 2 to 7 substitutions were observed in *NSP1*, *NSP3* and *NSP4*, *NSP5* was conservative.
Discussion. The results obtained are consistent with the literature data and indicate the participation of *NSP* genes in maintaining the heterogeneity of the rotavirus population.
Conclusion. Until 2018, the genetic diversity of rotaviruses in Nizhny Novgorod was determined by the circulation of strains carrying several alleles of the *NSP2* gene and conservative genes *NSP1*, *NSP3–NSP5*. By the end of the study period, new variants of the genotype G9P[8] were formed in the population, carrying previously unknown combinations of alleles of nonstructural genes.

Keywords: *rotavirus; nonstructural genes; phylogenetic analysis; genetic variants; amino acid substitutions*

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НАУЧНАЯ СТАТЬЯ
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Вариабельность генов неструктурных белков ротавируса А (Reoviridae: *Rotavirus: Rotavirus A*) генотипа G9P[8] в период доминирования на территории Нижнего Новгорода (центральная часть России) (2011–2020)

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Введение. В России ротавирус А является основной причиной тяжёлого гастроэнтерита вирусной этиологии у детей раннего возраста. Молекулярные особенности, позволяющие ротавирусу того или иного генотипа получить эволюционное преимущество, остаются неясны, поэтому изучение генетического разнообразия ротавирусов на основе генов, кодирующих неструктурные белки, ответственных за репродукцию вируса в клетке, является актуальной задачей.
Цель работы – изучение генетического разнообразия ротавирусов генотипа G9P[8], доминировавшего в Нижнем Новгороде в 2011–2020 гг., на основе генов, кодирующих неструктурные белки.
Материалы и методы. Ротавирус-положительные образцы стула детей исследовали методами ПЦР-генотипирования и секвенирования нуклеотидных последовательностей генов *NSP1–NSP5*. Филогенетический анализ проводили в программе MEGA X.
Результаты. В период 2011–2020 гг. в Нижнем Новгороде происходила коциркуляция ротавирусов G9P[8], имеющих четыре варианта гена *NSP2*. Новые аллели были отмечены в 2012 (N1-a-III), 2016 (N1-a-IV) и 2019 гг. (N1-a-II). Появление новых вариантов других генов произошло в 2014 (E1-3, *NSP4*), 2018 (T1-a3-III, *NSP3*) и 2019 гг. (A1-b-II, *NSP1*). Наиболее вариабельным по аминокислотной последовательности был *NSP2* (16 замен), для *NSP1*, *NSP3* и *NSP4* было показано от 2 до 7 замен, *NSP5* был консервативен.
Обсуждение. Полученные результаты согласуются с данными литературы и свидетельствуют об участии генов *NSP* в поддержании гетерогенности популяции ротавирусов.
Заключение. До 2018 г. генетическое разнообразие ротавирусов в Нижнем Новгороде определялось коциркуляцией штаммов, несущих несколько аллелей гена *NSP2*, и консервативными генами *NSP1*, *NSP3–NSP5*. К концу изучаемого периода в популяции сформировались новые варианты генотипа G9P[8], несущие ранее не встречавшиеся комбинации аллелей неструктурных генов.

Ключевые слова: *ротавирус; неструктурные гены; филогенетический анализ; генетические варианты; аминокислотные замены*

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Introduction

Rotaviruses (RVs, the genus *Rotavirus,* the family Reoviridae) are a major etiological agent of severe dehydrating diarrheas primarily in young children, accounting for 30 to 70% of hospitalizations for acute gastroenteritis in different countries [1]. The worldwide occurrence and stability of the pathogen in the environment, the vast variety of transmission factors and pronounced seasonality contribute to high incidence rates of rotavirus infection [2].

RVs have a segmented double-stranded RNA genome consisting of approximately 18,555 nucleotides in total [3, 4]. Having segmented genomes, RVs can undergo the exchange of segments (reassortment) between strains belonging to different genotypes, while infecting the cell, thus providing mechanisms for evolution and maintenance of RV genetic diversity [5].

Eleven segments of group A rotavirus (RVA) RNA encode 12 proteins. Six proteins are structural capsid components (VP1–VP4, VP6, VP7) participating in assembly of a viral particle and host cell invasion. The other six proteins are nonstructural and perform such important functions as suppression of apoptosis and host innate immune responses (NSP1), replication of the virus genome, capsid packaging and viroplasm formation (NSP2 and NSP5), inhibition of host protein synthesis (NSP3), acting as toxins causing dehydrating diarrheas (NSP4) [6].

RVs are characterized by broad antigenic and genetic diversity. Using the binary classification, at least 41 G genotypes (based on the VP7 glycoprotein gene) and 57 P genotypes (based on the VP4 protease-sensitive protein gene) have been identified in RVAs of humans and animals. In addition to the binary system, following the whole-genome classification, each segment of the virus genome can be assigned to a specific genotype [7]. To describe a complete genotype, the nomenclature Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx is used for genes encoding proteins VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/NSP6, respectively [5].

Currently, the most globally common six G[P] types of RVAs are G1P[8], G2P[4], G3P[8], G4P[8], G9P[8] and G12P[8] [8–11]. These RV genotypes are also well represented in Russia. G9P[8] representatives were first identified in infants in Omsk in 2002, and later in 2009, they were found in the European part of Russia (Moscow Region) [12–15]. In Nizhny Novgorod, this genotype was detected in the 2011–2012 season, accounting for 26.9%; by 2016, it became the dominant genotype (58.8%) [8, 16].

G9P[8] genotype rotaviruses were earlier characterized with reference to genes encoding VP7 and VP4, proteins participating in the virus entry to the cell [8, 13, 17]. High circulation activity of RVs can be maintained due to evolutionary changes in nonstructural proteins responsible for virus replication in the cell. The primary function of the NSP1 protein is inhibition of cellular apoptosis and suppression of innate immune responses [18]. The NSP2 protein participates in virus genome replication and capsid packaging; together with NSP5, it plays a critical role in the formation of viroplasms during maturation of viral particles [19]. NSP3 participates in the inhibition of host protein synthesis, inactivating two factors of translation initiation (eIF4F and eIF2), which are required for translation of mRNA (messenger RNA) of the host cell [20]. The NSP4 glycoprotein is a rotavirus enterotoxin and has immunogenic properties [21]. As known, the 11th genome segment encodes two RV proteins – NSP5 and NSP6. The open reading frame of the NSP6 gene is located between nucleotide positions 80–358 and encodes the NSP6 protein consisting of 92 amino acid (aa) residues [22]. The NSP5 protein is involved in virus genome replication, and together with NSP2, participates in the formation of viroplasms in infected cells. The functions of the NSP6 protein are not fully defined, though some findings suggest that it interacts with nucleic acids [23].

Therefore, **the objective of the study** was to explore the genetic diversity of G9P[8] rotaviruses, which prevailed in Nizhny Novgorod in 2011–2020, through genes encoding nonstructural proteins.

Materials and methods

The study was performed using fecal specimens collected from children hospitalized with symptoms of acute intestinal infection to the children's hospital of infectious diseases in Nizhny Novgorod during 2011–2020.

RIBO-prep and REVERTA-L reagent kits (Central Research Institute of Epidemiology of Rospotrebnadzor (CRIE, Russia) were used for extraction of nucleic acids and for the reverse transcription reaction. Rotavirus RNA was detected using AmpliSens Rotavirus/Norovirus/Astrovirus-FL testing system utilizing real-time polymerase chain reaction (PCR) (CRIE, Russia).

The G/P genotype of RVs was identified by multiplex PCR for identification of 12 genotypes of *VP4* and *VP7* genes, using a set of type-specific primers for genotypes G1–G4, G6, G8, G9, G12, P[4], P[6], P[8], and P[9] [24–29]. The PCR products were identified using agarose gel electrophoresis and ethidium bromide.

cDNA (complementary DNA) fragments were produced for sequencing during PCR using reagents manufactured by LLC "Sileks" (Russia) and primers synthesized at LLC "Syntol Scientific and Production Company" (Russia). The primer sequences are listed in the publication of Sashina et al. [13].

cDNA fragments of nonstructural *NSP1–NSP5* genes were sequenced for two strands using forward and reverse primers [13], the Dye Terminator Cycle Sequencing (DTCS) QuickStartKit (Beckman Coulter, United States), and Beckman Coulter CEQ 8000 genetic analysis systems (Beckman Coulter, United States) in accordance with the manufacturer's instructions.

The online BLAST program was used for search of related sequences. Nucleotide sequences of the Nizhny Novgorod RV strain genome, which were published earlier [14, 17] were retrieved for the phylogenetic analysis from the GenBank database, along with nucleotide sequences from other countries, which are available in

GenBank. The nucleotide sequences obtained in this study were deposited to GenBank with accession numbers MW842500–MW842550.

The alignment of nucleotide sequences, the phylogenetic analysis, and the analysis of deduced amino acid sequences were performed with MEGA X software [30]. The statistical assessment of the tree topology was performed by the bootstrap method using 1,000 random samples. The model of nucleotide substitutions for each alignment was selected using the Bayesian information criterion. The best-fitting model for *NSP1–NSP4* genes was the Tamura 3-parameter model, and for the *NSP5* gene – the Kimura 2-parameter model. Phylogenetic trees were constructed by the maximum likelihood method with MEGA X software [31].

The studied strains were assigned to phylogenetic lineages and sublineages by clustering isolates on the phylogenetic trees with a bootstrap support for nodes above 75% and high similarity of nucleotide sequences (97.0–100.0%). Phylogenetic lineages and sublineages were denoted in accordance with the commonly used classification [13, 17, 32–35].

The study was conducted with the informed consent of the legal representatives of minor patients. The research protocol was approved by the Local Ethics Committee of the Blokhina Nizhny Novgorod Research Institute of Epidemiology and Microbiology of the Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing (Rospotrebnadzor) (Protocol No. 6 dated March 24, 2021).

Results

A total of 13,777 fecal specimens from children hospitalized with the diagnosis of acute intestinal infection were tested during 2011–2020. Rotavirus RNA, which was further used for G[P] genotyping, was detected in 3,994 (28.9%) specimens. The G[P] type of RVs was identified in 2,736 (68.5%) specimens by PCR. Genotypes for 1,258 (31.5%) specimens were not identified.

Proportional contribution of G9P[8] rotaviruses in Nizhny Novgorod during 2011–2020

The proportional distribution of RVs with different genotypes during the studied period is shown in **Fig. 1** *a*.

The spectrum of PCR-identified G/P genotypes of RVs included 6 globally common genotypes (G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8]). Throughout the studied period, the proportional contribution of the G9P[8] genotype was 29.3%, and it was a dominant genotype in Nizhny Novgorod. The G4P[8] genotype strains rank second, accounting for 27.8%, and were followed by G2P[4] (8,8%), G1P[8] (8.6%), G3P[8] (1.8%), G8P[8] (0.8%), and G12P[8] (0.1%).

The proportional contribution of RVs with the G9P[8] genotype varied during different seasons. For the first time, these strains were detected in Nizhny Novgorod during 2011–2012 season, accounting for 26.9% (**Fig. 1** *b*). The next two seasons were characterized by low circulation levels of RVs with the G9P[8] genotype (1.0–3.2%), which significantly increased by the 2014–2015 season, reaching 35.6%. During the 2015–2016 season, RVs with this genotype accounted for 39.6%, having replaced the previously dominant strains with the G4P[8] genotype. The 2016–2017 season was notable for the highest proportion of representatives of the G9P[8] genotype, which accounted for 58.8%. By 2017–2018, their proportion decreased to 40.5%, followed by another decrease to 27.7% during the 2018–2019 season. In 2019–2020, the G9P[8] genotype regained its dominant positions, accounting for 57.2%.

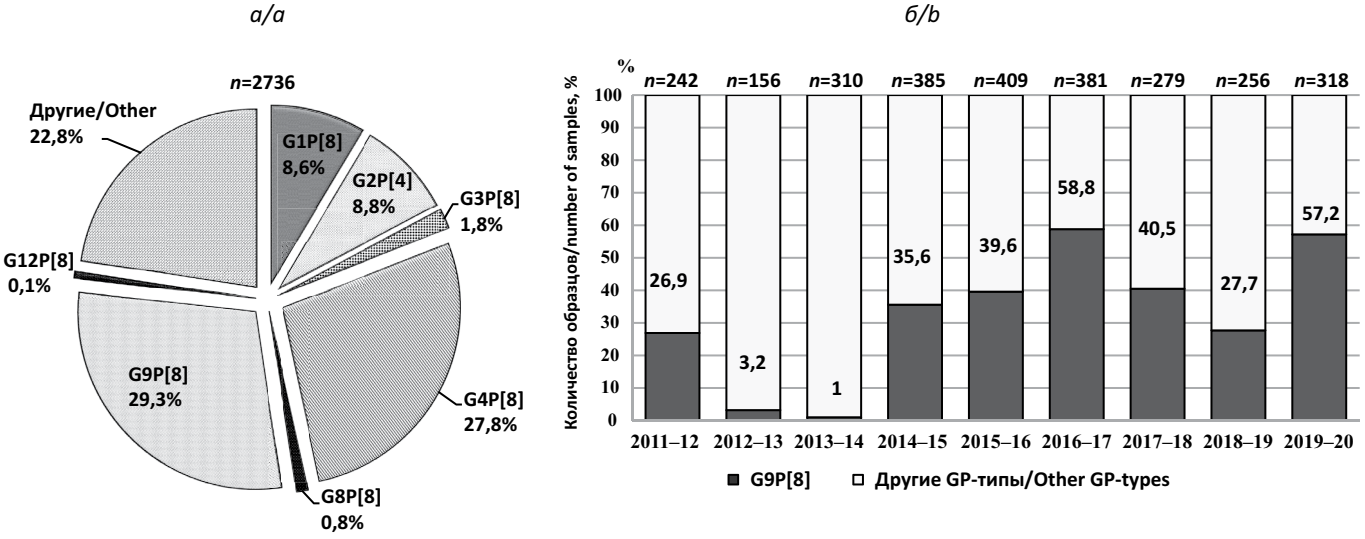

Fig. 1. Distribution of rotavirus A genotypes in Nizhny Novgorod in the period 2011–2020: *a* – the percent distribution of basic genotypes of rotaviruses in whole study period; *b* – the percent of genotype G9P[8] rotaviruses in different seasons of the period of study.

Рис. 1. Распределение генотипов ротавируса А в Нижнем Новгороде в период 2011–2020 гг.: *а* – долевое распределение ротавирусов основных генотипов за весь изучаемый период; *б* – долевой вклад ротавирусов генотипа G9P[8] в разные сезоны изучаемого периода.

The fluctuations in the proportional contribution of G9P[8] RVs could be associated with genetic rearrangements in the virus population, involving nonstructural genes.

Phylogenetic analysis of G9P[8] rotaviruses focusing on NSP1–NSP5 genes

A total of 16 strains isolated in different seasons during 2011–2020 were used for the molecular and genetic analysis of G9P[8] RVs focusing on genes encoding nonstructural proteins.

Fig. 2 a shows the abbreviated phylogenetic tree containing 86 nucleotide sequences of the RV NSP1 gene, including 21 sequences from Nizhny Novgorod.

RVs with the A1 genotype, which belong to two sublineages (A1-b-I and A1-b-II) of phylogenetic lineage A1-b, were identified in Nizhny Novgorod during the studied period. Most of the Nizhny Novgorod strains with the G9P[8] genotype, which were isolated throughout the studied period (86.7%), as well as the related RVs from Hungary, Germany, Italy, and Egypt, which had G1P[8], G4P[8], G9P[8], and G12P[8] genotypes, belonged to sublineage A1-b-I. The Nizhny Novgorod strains showed a 97.8–99.9% nucleotide sequence identity within the sublineage. Sublineage A1-b-I was also represented by 3 strains with the G4P[8] genotype from Nizhny Novgorod. Sublineage A1-b-II included 2 G9P[8] strains from Nizhny Novgorod, which were isolated in 2019–2020; the nucleotide sequence identity between them was 99.6%. The most closely related strains were RVA isolates with G9P[8] and G12P[8] genotypes from Pakistan and Paraguay. The difference between these strains and representatives of sublineage A1-b-I was 6.3–8.1%. The above sublineage also included 3 Nizhny Novgorod strains with the G1P[8] genotype [8].

107 nucleotide sequences, including 21 sequences from Nizhny Novgorod, were used for the analysis of the intragenotypic diversity of RVs with the reference to the *NSP2* gene. The adapted phylogenetic tree is shown in **Fig. 3** *a*. The analyzed RV nucleotide sequences had the N1 genotype; they belonged to one phylogenetic lineage N1-a and four sublineages (I–IV). The nucleotide sequence identity demonstrated by the *NSP2* gene of the strains within the sublineages was 99.7–99.9%. The difference among the representatives of different sublineages ranged from 4.8 to 10%.

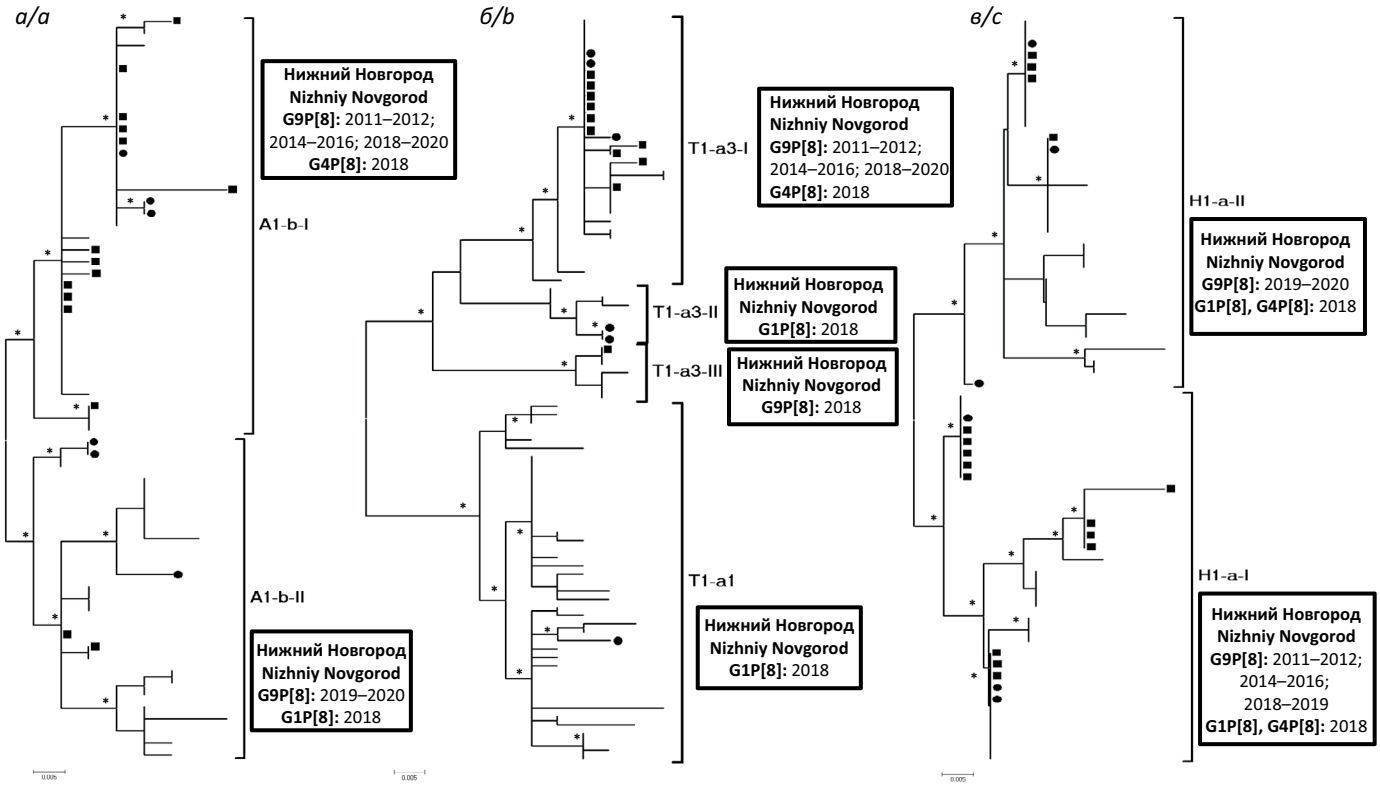

Fig. 2. Phylogenetic tree based on the nucleotide sequences: *a – NSP1*; *b – NSP3*; *c – NSP5* gene of rotavirus A strains.

A sign ■ the strains of the G9P rotavirus A genotype are marked G9P[8]; with a sign ● rotavirus A strains of other genotypes (G1P[8], G4P[8]) were noted.

*Node support index over 75.

Рис. 2. Филогенетическое дерево, построенное на основе нуклеотидных последовательностей гена: *а – NSP1*; *б – NSP3*; *в – NSP5* штаммов ротавируса А.

Знаком ■ отмечены штаммы ротавируса А генотипа G9P[8]; знаком ● отмечены штаммы ротавируса А других генотипов (G1P[8], G4P[8]).

*Индекс поддержки узла более 75.

Sublineage N1-a-I included 4 strains from Nizhny Novgorod, which were isolated in 2018–2019. The isolates with G1P[8] and G4P[8] genotypes from Nizhny Novgorod, Novosibirsk, and Omsk were most closely related to them. Sublineage N1-a-II included one Nizhny Novgorod strain with the G9P[8] genotype, which was isolated in 2019, as well as related RVs from Nizhny Novgorod, the United States, Italy, Japan, and Novosibirsk, which had G1P[8] and G3P[8] genotypes. Five G9P[8] strains from Nizhny Novgorod, which were isolated in 2012 and 2020, represented sublineage N1-a-III. The related isolates were from Hungary, Japan, and Novosibirsk, all of them having the G1P[8] genotype. The above sublineage also included one Nizhny Novgorod G1P[8] strain (2018). Sublineage N1-a-IV included 5 Nizhny Novgorod strains, which were isolated in 2016 and 2019, as well as the related G4P[8] RVs from Novosibirsk and Ethiopia, which were detected in 2012 and 2016.

The phylogenetic tree containing 89 nucleotide sequences of the *NSP3* gene, including 17 sequences from Nizhny Novgorod, is shown in **Fig. 2** *b*. The Nizhny Novgorod RVs with the G9P[8] genotype belonged only to one lineage (T1-a3), while 10 of 11 (90.9%) strains belonged to sublineage T1-a3-I. The nucleotide sequence identity among these strains was 99.1–99.6%. RVs that were closest related to them were those with G9P[8] and G4P[8] genotypes from Hungary and Thailand. The sublineage also included 3 Nizhny Novgorod strains with the G4P[8] genotype, which were isolated in 2018; however, their nucleotide sequences were identical to those of the strains only by 95.5–96.0%. One strain with the G9P[8] genotype, which was isolated in Nizhny Novgorod in 2018, belonged to sublineage T1-a3-III, which also included 2 Nizhny Novgorod RVs with the G1P[8] genotype. The difference between this strain and T1-a3-I was 8.1%.

Nucleotide sequences of 23 Nizhny Novgorod strains isolated in Nizhny Novgorod and 63 sequences from other countries were used for the analysis based on the *NSP4* gene. The phylogenetic tree is shown in **Fig. 3** *b*. The Nizhny Novgorod RVs had the E1 genotype and belonged to two phylogenetic lineages (E1-1 and E1-3).

Twelve strains with the G9P[8] genotype (70.6%) represented sublineage E1-1-I. The nucleotide sequence identity was 98.9–99.9%. The related strains were those with G1P[8] and G12P[8] genotypes from Australia, Germany, the United States, and Italy, which were isolated during 2008–2016.

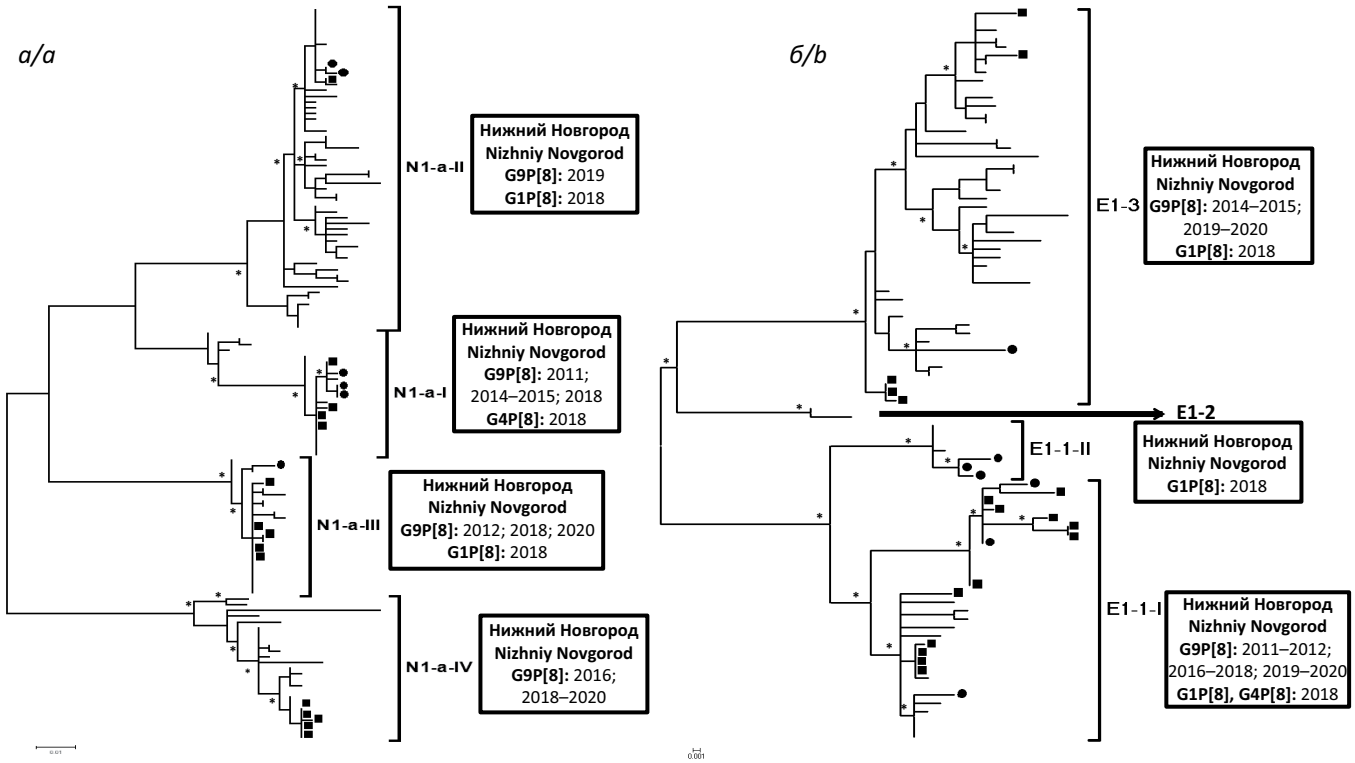

Fig. 3. Phylogenetic tree based on the nucleotide sequences: *a – NSP2*; *b – NSP4* gene of rotavirus A strains.
A sign ■ the strains of the G9P rotavirus A genotype are marked G9P[8]; with a sign ● rotavirus A strains of other genotypes (G1P[8], G4P[8]) were noted.
*Node support index over 75.

Рис. 3. Филогенетическое дерево, построенное на основе нуклеотидных последовательностей гена: *а – NSP2*; *б – NSP4* штаммов ротавируса А.
Знаком ■ отмечены штаммы ротавируса А генотипа G9P[8]; знаком ● отмечены штаммы ротавируса А других генотипов (G1P[8], G4P[8]).
*Индекс поддержки узла более 75.

Lineage E1-3 included 4 G9P[8] strains (29.4%) from Nizhny Novgorod, having the nucleotide sequence identity of 97.9–99.9%. The related strains included one Nizhny Novgorod strain with the G1P[8] genotype and RVs with G1P[8], G3P[8], and G9P[8] genotypes from Japan, Belgium, China, and Zimbabwe, which were isolated during 2010–2014. The nucleotide sequence difference among the representatives of clusters E1-1-I and E1-3 was 14.9%.

A total of 22 Nizhny Novgorod RVs were used for the analysis based on the *NSP5* gene. The phylogenetic tree containing 93 nucleotide sequences in total is shown in **Fig. 2** *c*. The strains with the G9P[8] genotype from Nizhny Novgorod had the H1 genotype; they belonged to one phylogenetic lineage H1-a and to two sublineages – H1-a-I and H1-a-II. The nucleotide sequence identity within the sublineages was 98.9%–99.4%, while the difference among the representatives of different sublineages ranged from 3.1 to 3.7%.

Sublineage H1-a-I included 12 Nizhny Novgorod strains with the G9P[8] genotype (2011–2019) and 3 strains with G1P[8] and G4P[8] genotypes. Their closest relatives were RVAs with G4P[8], G12P[8], and G1P[8] genotypes from Hungary, Japan, and Ethiopia, which were isolated in 2008–2016. Sublineage H1-a-II included 4 RVs with the G9P[8] genotype, which circulated in Nizhny Novgorod in 2019–2020, and 3 strains with other genotypes, which were isolated in 2018. The related strains had G1P[8] and G12P[8] genotypes and were isolated in Thailand, Italy, and Nicaragua during 2010–2014. The combinations of sublineages of genes encoding nonstructural proteins of the studied RV strains PB are shown in **Table**.

Throughout the studied period, the Nizhny Novgorod population demonstrated the co-circulation of four variants of the *NSP2 gene*; new alleles were detected in 2012 (N1-a-III), 2016 (N1-a-IV) and 2019 (N1-a-II). New variants of other genes emerged in 2014 (E1-3, *NSP4* gene), 2018 (T1-a3-III, *NSP3* gene), 2019 (A1-b-II, *NSP1* gene; H1-a-II, *NSP5* gene).

Analysis of deduced amino acid sequences of nonstructural proteins of rotavirus A with the G9P[8] genotype

The analysis of the amino acid sequence of the NSP1 protein showed that the representatives of sublineage A1-b-II, which joined the Nizhny Novgorod population in 2019, differed from the previously circulating RVs belonging to sublineage A1-b-I at 6 positions (76, 115, 119, 131, 165, and 199). The analysis of the amino acid sequence of the NSP2 protein of N1 strains belonging to 4 sublineages (N1-a-I, N1-a-II, N1-a-III, and N1-a-IV) demonstrated the variability at 16 positions (64, 75, 82, 97, 98, 100, 108, 118, 135, 143, 200, 218, 249, 253, 254, and 255). Among the representatives of different sublineages of the N1 genotype, the largest number of differences in the amino acid sequence (8 substitutions) was demonstrated by the strains of sublineage N1-a-III, which were present in the Nizhny Novgorod population in 2012, 2018, and 2020. RVs belonging to sublineage N1-a-I and circulating during 2011–2018 had 3 amino acid differences from other sublineages. The strains of sublineage N1-a-IV, which were isolated in Nizhny Novgorod in 2016, had 4 amino acid substitutions, while the isolate belonging to sublin-

Table. Genes *NSP1–NSP5/NSP6* sublineages combinations among G9P[8] rotaviruses in Nizhny Novgorod
Таблица. Сочетания сублиний неструктурных генов *NSP1–NSP5/NSP6* у ротавирусов генотипа G9P[8] в Нижнем Новгороде

Штамм Strain	Год выявления Isolation year	Сегмент генома Genome segment					
		VP7, VP4 [8, 13, 17]	NSP1	NSP2	NSP3	NSP4	NSP5/6
NN2626	2011	G9-III-d, P[8]-3.6 [8]	A1-b-I	N1-a-I	T1-a3-I	E1-1-I	H1-a-I
NN445	2012	G9-III-d, P[8]-3.6	–	N1-a-III	T1-a3-I	E1-1-I	H1-a-I
NN459	2012	G9-III-d, P[8]-3.6	A1-b-I	–	–	E1-1-I	H1-a-I
NN561	2012	G9-III-d, P[8]-3.6	A1-b-I	N1-a-III	–	E1-1-I	H1-a-I
NN2721	2014	G9-III-d, P[8]-3.6	A1-b-I	N1-a-I	T1-a3-I	E1-3	H1-a-I
NN414	2015	G9-III-d, P[8]-3.6	A1-b-I	N1-a-I	T1-a3-I	E1-3	H1-a-I
NN176	2016	G9-III-d, P[8]-3.6	A1-b-I	N1-a-IV	T1-a3-I	E1-1-I	H1-a-I
NN291	2016	G9-III-d, P[8]-3.6	A1-b-I	N1-a-IV	T1-a3-I	E1-1-I	H1-a-I
NN385	2018	G9-III-d, P[8]-3.6 [13]	A1-b-I	N1-a-III	T1-a3-I	E1-1-I	H1-a-I
NN386	2018	G9-III-d, P[8]-3.6	A1-b-I	N1-a-IV	T1-a3-I	E1-1-I	H1-a-I
NN568	2018	G9-III-d, P[8]-3.6	A1-b-I	N1-a-I	T1-a3-III	E1-1-I	H1-a-I
NN856	2019	G9-VI-e, P[8]-3.6 [17]	A1-b-II	N1-a-II	–	E1-3	H1-a-I
NN1217	2019	G9-III-d, P[8]-3.6	A1-b-I	N1-a-IV	T1-a3-I	E1-1-I	H1-a-II
NN1428	2019	G9-III-d, P[8]-3.6	A1-b-I	N1-a-IV	–	E1-1-I	H1-a-II
NN839	2020	G9-III-d, P[8]-3.3	A1-b-II	N1-a-III	T1-a3-I	E1-3	H1-a-II
NN877	2020	G9-III-d, P[8]-3.6	A1-b-I	N1-a-III	–	E1-1-I	H1-a-II

eage N1-a-II (2019) had two substitutions. The analysis of the amino acid sequence the NSP3 protein showed the variability of the representative of T1-a3-III sublineage at two positions (222 and 255) compared to T1-a3-I strains that were conserved throughout the studied period. Generally, RVs belonging to two phylogenetic lineages (E1-1-I and E1-3) were variable at 12 positions of the NSP4 protein. RVs belonging to lineage E1-3 and detected in the population in 2014–2015 and 2019–2020 had seven amino acid substitutions (D124E, K150R, V154I, I155V, V158I, S174N, S182V/I) compared to the strains belonging to lineage E1-1 (sublineage I) and circulating during the studied period.

The analysis of the amino acid sequence of the NSP5/NSP6 protein of representatives of sublineages H1-a-I and H1-a-II showed the variability at 6 positions (124, 153, 170, 182, 185, 195). However, no amino acid substitutions that would be different in the sublineages were found. All the substitutions were single (S124G, R153K, S170R, K182N, F185Y, A195S) and typical of strains of both sublineages, which were isolated in 2012 (3 strains), 2018 (3 strains), and 2019 (1 strain).

Thus, in RVs with the G9P[8] genotype, the largest number of substitutions was found in the amino acid sequence of the NSP2 protein (16 positions), which was variable throughout the studied period. The NSP1, NSP3, and NSP4 proteins had from two to seven substitutions typical of all representatives of specific sublineages. The NSP5 protein was the most conserved one: Substitutions at 6 positions were found in single strains.

Discussion

The published data show that RV strains with the G9P[8] genotype are well represented globally; in some countries, they have dominant occurrence. For example, Zhou et al. (2020) report that the G9P[8] genotype accounted for 74.5% in China during 2011–2019 [36]. The predominance of G9P[8] was reported in different regions of Italy, starting from 2008–2009 [37]. The 2012–2013 period was characterized by a high proportion of G9P[8] RVs in Moscow (30%), which were related to the Nizhny Novgorod and Turkish strains. Later, in 2015–2020, strains with this genotype prevailed in the overall genotype distribution, accounting for 37% [38]. In Nizhny Novgorod, G9P[8] RVs prevailed in 2011–2020. The proportional contribution of G9P[8] varied during different seasons, ranging from 1 to 58.8%.

Six phylogenetic lineages (I–VI) of the *VP7* gene were identified for the G9 genotype. Representatives of lineage G9-III are well represented worldwide [39]. On the phylogenetic tree, the Nizhny Novgorod RVs isolated in 2011–2016 grouped within sublineage G9-III-d into two clusters with strains from Novosibirsk (2011–2012) and Turkey (2014–2016) [8, 13]. For several years, sublineage G9-III-d was the only one in Nizhny Novgorod. After 2018, the Nizhny Novgorod population demonstrated the presence of G9P[8] strains belonging to sublineage G9-IV-e, which also included human RVs detected in China and porcine RVs isolated in Japan. The representatives of G9-III-d sublineage were conserved in their amino acid composition of the VP7 protein for a long time and differed from strains of sublineage G9-VI-e by one amino acid at position 100 (D100N). Phylogenetic lineage P[8]-3 of the *VP4* gene was most prevalent for the P[8] genotype in Russia. In 2016–2020, strains of sublineages P[8]-3.1, P[8]-3.3, and P[8]-3.6 were well represented in Nizhny Novgorod, Moscow, and Novosibirsk. At the same time, single representatives of P[8]-3.4 were detected in Nizhny Novgorod in 2017 and in Omsk in 2008 [17]. The strains used in this study had been described earlier and had alleles G9-III-d and P[8]-3.6 of *VP7* and *VP4* genes.

The genes of nonstructural proteins of G9P[8] RVs had the following characteristics: They remained conserved throughout most of the studied period and had new alleles (*NSP1*, *NSP3*, *NSP5*) by the end of the period or variable nucleotide sequences (*NSP2*, *NSP4*) during their predominance.

Allele A1-b-I of the *NSP1* gene was the only one among G9P[8] RVs circulating in Nizhny Novgorod during 2011–2018. Starting from 2019, the population was characterized by occurrence of strains belonging to sublineage A1-b-II and by their co-circulation with the strains carrying allele A1-b-I. The analysis of amino acid sequences of the NSP1 protein showed that the strains of sublineage A1-b-I had the conserved NSP1 protein, while compared to them, the strains belonging to sublineage A1-b-II had 6 substitutions. The amino acid substitutions in the NSP1 structure were located in two functional regions of the protein molecule: in the RNA-binding domain (S76N), the integrity of which is important for inhibition of apoptosis and implementation of innate immune protection mechanisms through degradation of interferon regulatory factors (IRF3) of the host, and in the region of interaction with the cytoskeleton (R115K, N119T, P131R, S165R), changes in which can result in translocation of the NSP1 protein to the cell nucleus (nuclear translocation of NSP1) [40]. One substitution (L199V) was located in the non-coding region.

Similar to the *NSP1* gene, for the *NSP3* gene, sublineage T1-a3-I was prevalent in Nizhny Novgorod and remained stable for at least 9 years. The emergence of the new allele (T1-a3-III) and genetic heterogeneity of G9P[8] RVs for the NSP3 gene were reported only in 2018. The representatives of sublineage T1-a3-I had the conserved NSP3 protein throughout the studied period. The only representative of sublineage T1-a3-III had two amino acid substitutions (I222V and A255V) compared to the strains belonging to sublineage T1-a3-I. The substitutions were located in the C-terminal domain of the protein molecule (205–313 aa), which is important for inhibition of translation of all eukaryotic mRNAs. This domain competes with the poly(A)-binding protein (PABP) for the segment of the eukaryotic translation initiation factor 4G [18, 41, 42].

For the *NSP5* gene, sublineage H1-a-I was also the only one among the Nizhny Novgorod G9P[8] RVs for 8 years. In 2019, the emergence of representatives of H1-a-II sublineage was reported in Nizhny Novgorod. No

differences in the amino acid sequence of NSP5 among representatives of different sublineages were found. Single amino acid substitutions were identified in some strains (2012, 2018–2019), which were located in the variable region (S124G, K143N), in the domain of interaction with NSP6 (S170R, K182N, F185Y, A195S) as well as in the homomultimerization domain (A195S) [43, 44].

As opposed to the genes discussed above, G9P[8] RV *NSP2* was characterized by variability of nucleotide sequences. A total of 4 alternating alleles of the gene were identified. In 2011, sublineage N1-a-I was the only one among G9P[8] RVs. In 2012, the emergence of representatives of sublineage N1-a-III was reported. In 2014–2015, RVs belonging to N1-a-I sublineage renewed their circulation to be replaced by strains of emerging sublineage N1-a-IV in 2016. In 2018, the co-circulation of G9P[8] RVs having three different alleles of the *NSP2* gene (N1-a-I, N1-a-III, N1-a-IV) was reported. In 2019, representatives of new sublineage (N1-a-II) co-circulated with N1-a-IV representatives, while in 2020, the only circulating strains were those belonging to sublineage N1-a-IV.

The variability of amino acid sequences of the NSP2 protein was highest among all the nonstructural proteins and affected 20 positions. Amino acid substitutions in the NSP2 structure were located in two functional regions of the protein molecule. Eight substitutions involved the N-terminal domain of the NSP2 monomer (S64N, I75N, A82N, N82I, E97D, I98V, N100S, and V108I). This domain contains single-stranded RNA-binding sites (1–97 aa). It is assumed that the interaction with RNA takes place in a loop consisting of 24 aa (52–76 aa). Conformational alterations in this region can affect the effectiveness of interaction between the protein and RNA. Twelve amino acid substitutions were located in the C-terminal domain (K118R, S135A, S143L, V200I, V218I, A249T, T249V, V249A, N253I, I254V, V254T, and T255I), which contains the nucleoside triphosphate attachment and hydrolysis site (109–317 aa). Substitutions in this region can also affect the effectiveness of the protein and RNA interaction [45].

Similar to the *NSP2* gene, clustering of Nizhny Novgorod strains for the *NSP4* gene took place within limited timespans. The strains isolated in 2011–2012 belonged to sublineage E1-1-I. In 2014–2015, representatives of lineage E1-3 came to the stage. Later, in 2016 and 2018, RVs belonging to sublineage E1-1-I renewed their circulation. The 2019–2020 period is characterized by the return of representatives of lineage E1-3 and their co-circulation with strains belonging to sublineage E1-1-I.

A total of 12 amino acid substitutions were identified in the primary structure of RV *NSP4*; the substitutions were located in three regions of the protein molecule. The T85I substitution was in the H3 domain (63–90 aa) involved in the protein retention in ER (the endoplasmic reticulum). The substitution (D124E) was located in the supercoiled domain (95–137 aa), which is important for oligomerization of the protein molecule of enterotoxin, its interaction with caveolin-1 and intracellular transportation of NSP4 from ER to the cell surface. Substitutions V158I, I166V, R167K, and S174N affected the site of interaction with the VP6 inner capsid protein (156–175 aa), which acts as an intracellular receptor for double-layered viral particles and is the key mediator in the viral morphogenesis. Other substitutions were located in interdomain regions [46].

Summing up the above, *NSP1*, *NSP3*, and *NSP5* genes were extensively conserved until 2018; then one new allele was identified in each of them. The *NSP2* gene demonstrated the highest variability among the genes encoding nonstructural proteins. Throughout the studied period, the Nizhny Novgorod population was characterized by co-circulation of its four variants; new alleles were reported in 2012 (N1-a-III), 2016 (N1-a-IV), and 2019 (N1-a-II). The study results correlate with the data provided by Ianiro et al. (2013) [37]. The analysis of nucleotide sequences of G9P[8] RVs identified differences ranging from 2 to 7% for different genes. The Italian strains had similar cluster formation on the phylogenetic trees based on *NSP1*, *NSP3,* and *NSP5* genes. The isolates were grouped into two clusters within A1, T1, and H1 genotypes. The *NSP2* gene demonstrated the highest variability and the lowest identity levels (92%). The strains comprised three clusters within the N1 genotype. The *NSP4* gene was most conserved: All the studied sequences were assigned to one cluster [29]. Thus, the findings support the role of nonstructural proteins participating in regulation of intracellular processes and in RV evolution, on an equal level with structural proteins.

Conclusion

The retrospective analysis of G9P[8] RVs was performed, involving genes encoding nonstructural proteins. During the 2011–2018 period, which included the predominance of the above genotype in 2015–2017, the genetic diversity of RVs was reached through the co-circulation of strains carrying different alleles of the *NSP2* gene (sublineages N1-a-I, N1-a-III, N1-a-IV). The amino acid sequence of NSP2 had 16 substitutions that were detected in functional regions, which participate in interaction with RNA and hydrolysis of nucleoside triphosphate.

The 2018–2020 period demonstrated a decline in the circulation of G9P[8] RVs and was characterized by the emergence of new alleles of *NSP1*, *NSP2*, *NSP3*, and *NSP5* genes (sublineages A1-b-II, N1-a-II, T1-a3-III, H1-a-II) and by the re-emergence of the allele of the *NSP4* gene belonging to lineage E1-3. The population had new variants of the G9P[8] genotype, which carried new allele combinations of all nonstructural genes.

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